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The effects of heat stress in redox balance and inflammatory signaling in porcine skeletal muscle

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ABSTRACT

Heat stress (HS) is a major concern for human health as well as for the swine industry. In 2012, HS resulted in the largest number of weather related human fatalities in the US. Animal production is also compromised as HS leads to poor sow performance, decreased carcass value and increased veterinary costs. However, the molecular effects of HS on skeletal muscle are still unclear. This study aimed to determine the extent to which HS disrupted redox balance and initiated an inflammatory response in oxidative and glycolytic porcine skeletal muscle. Moreover, we quantified the contribution of reduced feed intake to the disruption of redox balance and inflammatory signaling in porcine skeletal muscle. To achieve this, crossbred gilts were subjected to thermoneutral (TN; 20°C) or HS (35°C) conditions for 1, 3 or 7 days. In order to quantify the contribution of reduced feed intake to the HS response, a group of 7 day TN animals were pair fed (PFTN) to the 7 day HS group. One day of HS increased oxidative stress, measured as a 1.5 fold increase in MDA modified proteins in oxidative muscle but not glycolytic muscle. Further, HS significantly increased the activities of catalase and SOD in oxidative muscle. These data indicated that oxidative muscle was more sensitive to HS than glycolytic muscle. Further, inflammatory signaling was not increased as a result of the HS treatment in STR or STW muscle. Also, reduced feed intake did not significantly contribute to increased oxidative injury or inflammatory signaling in porcine skeletal muscle.

CHAPTER I

LITERATURE REVIEW

Global Warming

It is well established that average temperatures around the world have increased in the last 100 years and are predicted to continue to rise at an alarming rate (37, 63, 70). Current estimates indicate that Earth's global average air temperature has risen 0.7°C in the last decade alone (63). Though the exact cause of climate change is unknown, some of the factors that contribute to global warming include natural causes such as changes in the sun's energy or shifts in ocean currents, and human emissions of greenhouse gasses (27). Research shows that human activities explain most of the recently observed warming (62). Projections for the US are that by the year 2100, temperatures will increase anywhere from 2.3°C to 6.3°C (85). These projections imply then that there will be more frequent intense heat events or heat waves. As a consequence, humans, animals and plants will be more prone to heat-related illnesses, accentuating the need to better understand cellular and physiological responses of organisms to heat stress.

Heat Stress

Heat stress (HS) results from the inability of an organism to properly balance the amount of energy produced and/or absorbed from the environment to the amount of energy being dissipated or released back into the environment (78). Every organism has a set thermoneutral (TN) zone, defined as the temperature range at which metabolic heat production and evaporative heat loss are relatively steady (45). When organisms are exposed to temperatures above this range they experience HS.

Heat Stress and Human Health

HS has many negative implications in human health. Among the most prominent heat-related illnesses experienced by humans are heatstroke, heat exhaustion, heat syncope, and heat cramps (44). In extreme cases, heat stroke in particular, can lead to death. In 2012, heat-related illnesses resulted in the largest number of weather related fatalities in the US; combining deaths due to tornadoes, hurricanes, lightning, and floods still did not add up to the number of lives claimed by HS (60). Further, in a two-week heat wave in 2003 in Europe, nearly 50,000 deaths were reported (47).

Though heat-related illnesses present a threat to human welfare worldwide, there are currently no treatments in place to alleviate heat-stress. Currently, most recommendations or methods applied aim to prevent HS, as opposed to treat it. One of the few methods or treatments today applied to heat-stressed individuals is removal from the heat environment, cooling and rehydrating (14). The urgency to find effective interventions for heat-related illnesses is underscored by the expanding world population, which is expected to surpass the 9 billion mark by the year 2050 (22). Moreover, most of this population growth is expected to occur in developing countries (83), most of which are found in the tropical and subtropical areas of the world, exposing greater numbers of people to HS (15).

Heat Stress and Animal Agriculture

Animals produced for human protein needs, such as pigs, cattle and chickens, like all other organisms, posses a set TN zone. When ambient temperatures deviate from this TN zone, production parameters are affected negatively, leading to great economic

losses (78). Some of these production parameters include, but are not limited to average daily gain, litter size, and lean meat percentage of the carcass. As stated before, HS animals exhibit compromised production parameters; among the most affected production parameters are milk yield and composition (fat, protein, and lactose content), reproduction, carcass value (which includes lean cuts, trimmable fat and bone) and lean growth (64, 72, 78). As a consequence of heat-related illnesses in production animals, there is also an increase in veterinary costs, which further contributes to the HS economic burden. It is estimated that the US animal agriculture industry loses approximately $900 million per year in dairy and more than $300 million per year in beef and swine as result of HS (78).

In Iowa, the largest producer of finishing hogs and sows in the US, losses sum up to around $5 million as a direct result of HS (78). With more extreme summers and large percentages of humidity, animals in Iowa are more disposed to experience the effects of heat stress. Even with advancements in cooling systems and housing facilities HS continues to negatively affect the economic aspect of animal production.

However, economic losses are not the only concern for animal producers. With the expected increases in population growth, there will be an approximate 60% increase in demand for food (28). Thus, there is a need to better understand the physiological and cellular changes associated with HS in order to develop solutions to better mitigate its negative effects.

Heat Stress Effects on Pig Physiology

It is a common observation that pigs exposed to chronic HS have reduced weight gain and increased respiration rates (64, 65, 78). Pigs employ multiple mechanisms to dissipate heat in an attempt to decrease their body temperatures. One of the most frequently occurring one is a reduction in feed intake. HS decreases feed intake by approximately 50% (21). This decreased nutrient intake can explain some, but not all, of the weight difference between animals in a HS environment compared to an animal in a TN setting (48). Decreased feed intake allows the pig to reduce its metabolic activities, thus decreasing the amount of heat produced as a result of digestion and absorption of food nutrients.

HS also causes increased respiration rates in pigs (1, 64) and results in panting, the major heat dissipation mechanism employed by pigs. Pigs posses very few or no sweat glands, thus they rely heavily on panting to dissipate heat (1, 31). However, panting is not a very effective heat dissipation mechanism, as it requires a great deal of skeletal muscle activity, which in turn produces heat (30). This might partly explain why pigs are more susceptible to heat stress when compared to other mammals, such as humans, with greater numbers of sweat glands used for temperature adjustment.

Nonetheless, pigs and humans alike experience other afflictions associated with HS. One of these major afflictions is an increase in tight junction permeability in the gut (65). Increased tight junction permeability allows endotoxins commonly found in the gut to enter the blood stream. This, in turn, can lead to endotoxemia or death, as it is very difficult to treat (49). Indeed, increased LPS levels in plasma have been previously

reported in other heat stress experiments with pigs (65). Among other consequences, increased LPS can result in the initiation of a systemic inflammatory response (56, 68).

Rodent and bovine models of HS have demonstrated that HS, independent of feed intake, leads to a shift in energy metabolism substrate (5, 82), where non-esterified fatty acids are substituted by glucose as the main substrate for energy production (5, 72). Thus, there is an increase reliance on glycolysis as the main mechanism for ATP production. This observation is supported by increased insulin plasma levels in HS rodents, pigs and cows (71, 82, 89).

Reactive Oxygen Species

Reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radicals, superoxide and nitric oxide, are oxygen-containing molecules that are chemically reactive. ROS contain an unpaired electron in their outermost shell of electrons allowing them to quickly react with other molecules to achieve a stable configuration. When present in large amounts in a cell, ROS can cause oxidative damage to proteins, lipids, and DNA. Oxidative damage occurs when cells are unable to detoxify ROS and/or repair damaged proteins, lipids and DNA. Depending on the level of damage, proteins may lose functionality and are thus targeted for degradation. However, ROS can also serve as a signaling molecule that is required for some cell processes (42, 80) and is a normal product of aerobic respiration. During normal conditions, ROS can be found in micromolar levels in a cell. When ROS production exceeds normal levels, cells start to experience oxidative stress (88). Oxidative stress is commonly defined as a disruption of redox signaling and control within a cell (41). The resulting imbalance in pro-oxidant

and antioxidant environment, when not adequately restored, can have deleterious effects for cells. The increased production of free radicals, or reactive oxygen species, leads to oxidative stress that can be triggered by a wide variety of signals and/or stressor events, including but not limited to, drug metabolism, overexpression of ROS-producing enzymes, radiation, and metabolic disorders (33).

Free radicals, such as superoxide anion, can be produced by a variety of different sources including mitochondria, xanthine oxidase and NADPH oxidases. Mitochondria are the primary source of free radicals within a cell (4, 6, 10). Complexes I and III are the main sources of mitochondrial superoxide production as these are the sites where electron leakage most commonly occurs (26, 34). These electrons are then available to convert O_2 to the superoxide anion radical. This superoxide radical is produced during normal physiological conditions by the mitochondria as a byproduct of respiration (2). The dismutation of the superoxide radical, which can be spontaneous or enzymatic, converts it to H_2O_2. In turn, H_2O_2 can then form hydroxyl radicals, a highly reactive radical species, which is among the primary sources of oxidative injury in a cell (9).

NADPH oxidase can also contribute to the intracellular production of free radicals. In fact, the main role of NADPH oxidase is ROS generation and redox signaling within a cell (50). NADPH oxidase can produce the superoxide radical by catalyzing the transfer of an electron from NADPH to O_2. Also, xanthine dehydrogenase and xanthine oxidase, isoenzymes of xanthine oxidoreductase can also produce free radicals in a cell. Xanthine oxidase is often identified as the main producer of superoxide through reduction of O_2, while xanthine dehydrogenase has a greater affinity

for reducing NAD^+, though it can also reduce O_2 at a lower affinity (7, 55). Thus, both enzymes can generate the superoxide radical within a cell.

After 12, 16 and 18 hours of HS, increased levels of oxidative stress have been reported in skeletal muscle of chicken (43, 57). Chronic HS (6 months) also increased oxidative injury in amphibian skeletal muscle (92). Oxidative stress has been implicated with increased protein degradation through several mechanisms, including the activation of proteases (52, 53, 77, 90) and increasing the expression of genes involved in autophagy and proteolysis (24, 53). Moreover, ROS have been shown to impair protein synthesis by preventing mRNA translation (76, 93). The combination of increased protein degradation and decreased protein synthesis results in impaired muscle growth and decreased protein accretion (38).

Antioxidant Enzymes

Antioxidant enzymes metabolize ROS during normal cell activity. There are three main antioxidant enzyme families present in cells: catalase, superoxide dismutase and glutathione system. Each of these catabolizes specific chemical reactions that help eliminate free radicals within a cell.

Superoxide Dismutase

The superoxide dismutase enzyme family consists of three members (MnSOD, CuZnSOD and FeSOD) classified by their metal content. CuZnSOD is primarily found in the cytosol, though all three members can be found at cytosol at dissimilar concentrations (8, 29). Further, MnSOD is usually localized in the mitochondria and

FeSOD in the extracellular space (74). All three enzymes catalyze the dismutation of the superoxide radical to hydrogen peroxide and molecular oxygen. Increased MnSOD expression, in particular, is associated with increased ROS production by the mitochondria (46). Additionally, heat stress and heat treatment have been shown to increase total SOD activity in skeletal muscle of rodents and chickens (32, 75).

Catalase

The reaction of the SOD enzymes produces H_2O_2, which can degrade into the more toxic OH radical, hence ridding the cell of H_2O_2 is a high priority. Catalase catalyzes the reduction of hydrogen peroxide into water and oxygen. Catalase is localized in the peroxisomes of mammalian cells (54) as well as the cytosol (58). This enzyme has one of the highest turnover rates of all enzymes, though it's capacity is limited at low concentrations of H_2O_2 as its activity requires two H_2O_2 molecules to bind to its active site (20). Catalase is among the enzymes activated in response to oxidative stress. Moreover, therapeutic hyperthermia of skeletal muscle has been shown to increase levels of catalase activity, and other antioxidant enzymes, reducing oxidative stress (75).

Additional Antioxidant Systems

The glutathione system represents another family of antioxidant enzymes present in cells (84). Glutathione is present in cells in both, its reduced form (GSH) or its oxidized form (GSSG). In the oxidation reaction of GSH to GSSG, glutathione peroxidase catalyzes the reduction of hydrogen peroxide to water (86). On the other hand, in the reduction of GSSG back to GSH, glutathione reductase catalyzes the

oxidation of NADPH to NADP. There are numerous types of glutathione peroxidases in mammalian cells, and these are commonly found in the cytosol and mitochondrial matrix.

Additionally, cells exploit non-enzymatic antioxidants in order to quench free radicals. Among the most common, non-enzymatic free radicals are β-carotene, vitamin C and vitamin E. Non-enzymatic antioxidants represent the first line of defense during events of oxidative stress (11).

Inflammation

Inflammation is a highly regulated response triggered by stimuli such as infection or tissue injury. Bacterial infections can trigger an inflammatory response by binding to toll-like receptors found in the innate immune system (18, 25, 39, 69). The inflammatory response is then mediated in resident macrophages and mast cells through transcription factors such as nuclear factor kappa-B (NFKB) and activator protein 1 (FOS/JUN), which promote the production of cytokines and chemokines to aid in the resolution of the infection or injury. Cytokines are the primary regulators of the inflammatory response (39, 73).

Previous reports have shown that inflammatory signaling can be activated by increases in ROS (40, 51). ROS act as signaling molecules that trigger cytokine production (12, 59, 94) through activation of the NF-κB pathway. Moreover, inflammatory signaling can also be activated by lipopolysaccharides (LPS) (18, 56). LPS is increased in HS animals as a result of compromised intestinal permeability (64,

65). Consequently, TLR4 receptors in cells can recognize the bacteria and initiate an inflammatory response via NF-κB (39).

Activation of the NF-κB transcription factor has been associated with increased production of TRIM63, a muscle-specific ubiquitin ligase, as well as muscle atrophy (13, 36). That both increased plasma endotoxin and oxidative stress are present during HS further implicates inflammation as a potential contributor to impaired muscle growth during HS.

Nuclear Factor kappa-B Signaling

NF-κB is a redox sensitive transcription factor triggered by sudden changes in the cellular environment (91). It regulates the expression of genes involved in inflammatory responses, stress responses, growth factors, and apoptosis, among others (19). NF-κB is a member of the Rel family of proteins that exist in the cytosol of cells as homo- or heterodimers (23, 61) bound to IκB. When NF-κB dimers are bound to IκB they are unable to localize to the nucleus to initiate transcription (3). Upon stimulus by molecules such as tumor necrosis factor alpha (TNF-α), LPS, and/or ROS, which bind to TLRs in the cell membrane (69), a signaling cascade is initiated and the IκB kinase (IKK) complex is activated (81). IKK in turn phosphorylates IκB, which is then ubiquitinated and degraded by the proteasome. By removing the inhibitory IkB the NF-κB dimer is then free to translocate to the nucleus and initiate transcription of target genes.

For some time it has been appreciated that skeletal muscle produces IL-6 in response to exercise stimulus (67, 79). More recenlty, more muscle-produced cytokines

were discovered and termed “myokines”. Myokines can perform in an autocrine, paracrine or endocrine fashion, making muscle the largest endocrine organ in the body (35, 66). Further, NF-κB activity has been shown to increase with exercise, inducing the production of myokines in skeletal muscle (87). NF-κB signaling has also been reported in skeletal muscle in response to injury (17) and disuse (24) indicating that skeletal muscle is capable of initiating an inflammatory response.

CHAPTER II

HEAT STRESS LEADS TO REDOX IMBALANCE IN PORCINE SKELETAL MUSCLE

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Abstract

In addition to mortalities, heat stress (HS) is associated with myriad maladaptions including muscle dysfunction and impaired growth across species. Despite this common observation, the molecular effects of HS on pig skeletal muscle remain unclear. The purpose of this study was to determine the extent to which HS disrupted redox balance and initiated an inflammatory response in oxidative and glycolytic skeletal muscle. To achieve this, female pigs (5-6/group) were subjected to thermoneutral (TN; 20°C) or HS (35°C) conditions for 1 or 3 days and the semitendinosus removed and dissected into red (STR) and white (STW) portions. After 1 day of HS, relative abundance of proteins modified by malondialdehyde (MDA), a measure of oxidative damage, was increased 2.5 fold compared to TN in the STR but not the STW, before returning to TN conditions following 3 days of HS. This observation corresponded with increased catalase and MnSOD gene expression ($P<0.05$) and MnSOD protein abundance ($P<0.05$) in the STR but not the STW. In the STR catalase and SOD activity were increased by ~30% and ~130%, respectively, after 1 day of HS and returned to TN levels by day 3. One or 3 days of HS did not increase inflammatory signaling in the STR or STW. These results suggest that oxidative muscle is more sensitive to HS than glycolytic muscle, resulting in oxidative stress rapidly countered an antioxidant response. Given the early response, emphasis of mitigation strategies should focus on early events occurring during HS.

Keywords: oxidative stress, free radicals, inflammation, mitochondria, pig

Introduction

Heat stress (HS) results from the inability of an organism to properly dissipate heat energy produced (3, 50). In 2012, heat-related illnesses resulted in the largest number of weather related fatalities in the U.S. due to heat stroke (40) and also caused additional morbidities related to excess heat load (53). In addition to deleterious health consequences in humans, HS compromises animal agriculture production by suppressing growth of muscle tissue, among other deleterious effects, which has negative economic consequences (50). Given that the Earth's average temperature is predicted to continue rising (41), these problems will likely become more frequent and severe.

While phenotypically HS leads to mortality in humans and other animals and has been shown to negatively impact skeletal muscle growth in production species (9, 54), the mechanism leading to compromised muscle growth is largely unknown. This is of particular interest as hyperthermia has been used as an intervention to spare muscle from loss due to disuse (38, 47) as well as augment regrowth following atrophy (18, 46). In these studies hyperthermia was also found to decrease oxidative damage. In contrast, HS has been associated with increased production of reactive oxygen species (ROS) in avian skeletal muscle subjected to HS for 12 or 18 hours and 3-9 days, respectively (2, 26, 35). Oxidative stress has been shown to lead to protein degradation through increased proteolysis and autophagy (13, 31, 32, 49, 56). Moreover, ROS have been shown to impair protein synthesis by preventing translation (48, 58). The combination

of increased protein degradation and decreased protein synthesis results in impaired muscle growth and decreased protein accretion.

Another potential contributor to decreased muscle growth during HS is inflammation. ROS have been implicated with increased inflammatory signaling via activation of the NF-κB pathway (25, 29) by acting as signaling molecules (7, 39, 59). Independently, inflammatory signaling can also be activated by endotoxemia. As a result of compromised intestinal permeability (43, 44) bacterial lipopolysaccharide (LPS) can enter the circulation and can be recognized by TLR4 receptors on muscle cell membranes (14), initiating an inflammatory response via *NFKB* signaling (24). Activation of the NFKB transcription factor has been associated with increased production of ubiquitin ligases, as well as muscle atrophy (8, 22).

Given the potential importance of oxidative stress and inflammation as negative regulators of muscle growth, the purpose of this study was to determine the extent to which HS contributes to oxidative stress and inflammation in porcine skeletal muscle. Due to their anatomical and physiological similarities with humans (52) and high sequence homology to the human genome (21), pigs have long been used as biomedical models (5, 17, 33). We hypothesized that HS will result in a progressive increase in oxidative stress and inflammatory signaling in porcine skeletal muscle.

Materials and Methods

Study Design and Animal Treatments

All animal experiments were approved by the Iowa State University Institutional Animal Care and Use Committee. Prior data from these animals and a detailed study design have been previously reported (43). Female pigs (35±4 kg BW; n=5-6/group) were held at thermal-neutral (TN) conditions (20 ± 1°C; 35-50% relative humidity) or exposed to constant heat stress (HS) (35 ± 1°C; 20-35% relative humidity) for a period of 1or 3 days. Animals were sacrificed by the captive bolt technique followed by exsanguination at the end of the respective treatment period. At this time, the semitendinosus (ST) muscle was removed and dissected into red (STR) and white (STW) muscle. The red and white portions of the pig ST muscle are visually apparent; to assure sample purity only 2 gram sections were collected. Muscle samples were frozen in liquid nitrogen for further analyses.

Protein Abundance

In order to measure protein abundance, approximately 50 mg of muscle was powdered on dry ice and homogenized in 1.5 mL of protein extraction buffer (10 mM sodium phosphate, pH 7.0, and 2% SDS) using a Dounce homogenizer. The sample was then centrifuged at 1500 x g for 15 min at 20 °C to remove cellular debris. Protein concentration was then measured colorimetrically using a commercially available kit (Pierce® BCA microplate protein assay kit, Pierce, Rockford, IL). Samples were diluted to 4 mg/ml in loading buffer (62.5 mM Tris (pH 6.8), 1.0% SDS, 0.01% bromophenol blue, 15.0% glycerol, and 5% β-mercaptoethanol). Ten microliters (40 μg of protein) of

each sample were loaded into 4-20% precast gradient gels and proteins were separated at room temperature for 30 min at 60 V followed by 50 min at 120 V. Afterward, proteins were transferred (90 min; 90 V) to a nitrocellulose membrane with a pore diameter of 0.2 μm. Membranes were blocked in 5% dehydrated milk TTBS (Tris-buffered saline containing 0.1% Tween 20) solution for 1 hour and exposed to primary antibody overnight at 4°C in 1% dehydrated milk TTBS solution as follows: catalase (Sigma, primary 1:1000, cat. no C0979, secondary 1:2000), Malondialdehyde (Abcam; primary 1:5000, cat. no ab27642, secondary 1:2000), MnSOD (Abcam; primary 1:5000, cat. no ab13533, secondary 1:2000), NF-κB p-65 (Abcam; primary 1:1000, cat. no ab7970, secondary 1:2000), IL-6 (Abcam; primary 1:1000, cat. no ab6672, secondary 1:2000), phospho-NF-κB p65 (Thermo Scientific; primary 1:1000, cat. no MA5-15160, secondary 1:2000), TNF-α (Abcam; primary 1:1000, cat. no ab6671, secondary 1:2000), IκB-α (Santa Cruz Biotechnology; primary 1:1000, cat. no SC-371, secondary 1:2000). After three 10 min washes with TTBS, membranes were exposed to secondary antibody (as noted above) for one hour at room temperature in 1% dehydrated milk TTBS solution. Membranes were washed again 3 times for 10min with TTBS and detection was performed by enhanced chemiluminescence and X-ray film. X-ray film was then scanned and blot signal was quantified through the use of Kodak software (Rochester, NY). Optical density was determined and values for each group were normalized to the mean of TN samples on each membrane. Values are reported relative to TN. All membranes were stained with Ponceau S to assure equal loading. We found that Ponceau S staining was similar for all groups for all membranes.

mRNA Transcript Abundance

RNA was isolated using the TRIzol reagent according to the manufacturer's instructions (Invitrogen; Carlsbad, CA; cat. no 15596). Briefly, powdered muscle was homogenized with TRIzol reagent, centrifuged and extracted with chloroform, and precipitated with ethanol. Total RNA was DNase treated (RNaseFree DNase set, Qiagen Inc., Valencia, CA; cat. no 79254) and purified using a column (RNeasy kit, Qiagen Inc.; cat. no 74106) to remove potential genomic DNA contamination. RNA concentration and purity was determined by measuring absorbance at 260 nm and 280 nm with a Nanodrop (Thermo Scientific**,** Waltham, MA). Total RNA (1µg) was then reverse transcribed (Qiagen Inc.; cat. no 205311) and gene expression measured through qRT-PCR using SYBR green (Qiagen Inc.; cat. no 204056). Transcript abundance was determined by the delta CT method using 18S rRNA as the control gene, and fold change calculated from the delta delta CTs. Transcript abundance is presented as fold changes relative to TN. Sequences of primer pairs can be found in Table 1.

Enzymatic Activities

Catalase activity was measured according to manufacturer instructions (Catalase Assay Kit, Cayman Chemical Company, Item No. 707002). Catalase activity is measured colorimetrically from tissue homogenates (50 mM potassium phosphate, pH 7.0, 1mM EDTA) and determined by linear regression using a standard curve. The principle of the

assay is based on the peroxidatic function of catalase. Catalase reacts with methanol to produce formaldehyde, which is then measured spectrophotometrically. Catalase activity is expressed as nmol/min/mL/mg of protein. Total superoxide dismutase (SOD) activity was measured using a commercially available kit (Superoxide Dismutase Assay Kit, Cayman Chemical Company, Item No. 706002) according to manufacturer instructions. Like above, measured activity was determined colorimetrically from tissue homogenates (20 mM HEPES buffer, pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose) using linear regression from a standard curve. The principle of the assay is based on the dismutation of superoxide radicals produced by xanthine oxidase and hypoxanthine by all types of SOD. Total SOD activity is expressed as U/mL/mg of protein.

Statistics

As expected, TN groups were similar to each other for all measures as detected by a Student's T-test. Because of this the TN group used for analyses was comprised of equal representatives from both the 1 day TN and 3 day TN groups chosen randomly for each measurement. To determine the extent to which heat stress altered variables over time data from TN, 1 day HS, and 3 day HS animals were compared using an ANOVA followed by a Newman-Keuls post hoc test when appropriate. To determine statistical significance, α level was set at $p < 0.05$. Values are displayed as means ± SEM unless otherwise noted.

Results

Oxidative Stress

To determine the extent to which heat stress caused free radical damage in skeletal muscle, abundance of proteins modified by malondialdehyde (MDA), a marker of lipid peroxidation, was measured in STR and STW. In STR the relative abundance of proteins containing MDA adducts, was increased 2.5-fold compared to TN ($p<0.05$) after 1 day of HS (Figure 1A), however, returned to TN levels in the 3 HS group. More detailed analyses (single band analysis, band subgroups) demonstrated that this increase was due to a subtle increase in abundance of most or all bands rather than significant modifications in one or a small subset of proteins. HS did not increase MDA accumulation in STW (Figure 1B). To assure that the band at approximately 45 kDa was not the result of nonspecific binding from our secondary antibody we probed a membrane with secondary only and found that no such band was present. This supports the notion that the band detected is the result of MDA modified protein.

Given that free radical injury can be caused by a failure of the antioxidant system to adequately respond to an oxidative insult we measured transcript abundance of select antioxidant enzymes. In STR, mRNA transcript abundance of *CAT* and *SOD2* was increased by 4 fold and 1.5 fold, respectively, compared to TN ($p<0.05$) following one day of HS (Figure 2A). This increase in transcript abundance was transient as abundance for both *CAT* and *SOD2* returned to TN levels following 3 days of HS.

Following that general pattern of a rise and subsequent fall, transcript abundance of *SOD1*, was statistically similar to TN after 1 day of HS, however, was decreased by 25% ($p<0.05$) after 3 days of HS compared to TN (Figure 2A). As changes in mRNA transcript abundance of *CAT* and *SOD2* closely mirrored that of oxidative damage we measured protein abundance of catalase and MnSOD. In STR catalase protein abundance was similar between all treatment groups (Figure 3A), however MnSOD was increased 1.5 fold ($p<0.05$) after 1 and 3 days of HS compared to TN (Figure 3A). In the STR, activities of both catalase (Figure 4A) and SOD (Figure 4C) were increased by 30% and 130% ($p<0.05$), respectively, following one day of HS compared to TN but returned to TN levels after three days of HS.

An absence of apparent oxidative damage in STW could stem from a robust response in antioxidant enzyme expression and activity serving to mitigate ROS. Therefore, we measured antioxidant mRNA transcript and protein abundance and activity in STW. In general, the pattern of change observed in antioxidant enzyme transcript abundance in the STW followed closely that observed in STR in that there was a shift toward increased abundance following one day of HS followed by a reduction in antioxidant enzyme transcript abundance following three days of HS (Figure 2B). Specifically, in the STW *CAT* transcript abundance was similar following one day of HS, however was decreased 40% ($p<0.05$) after 3 days of HS compared to the 1 day HS group, however failed to reach significance compared to TN. *SOD1* was also decreased after 3 days of HS by 40% and 50% compared to TN and 1 day of HS, respectively ($p<0.05$). Finally, *SOD2* transcript abundance was increased by 40% ($p<0.05$) after 1

day of HS compared to TN, but decreased ($p<0.05$) after 3 days of HS by 30% and 50% compared to TN and 1 day of HS, respectively (Figure 2B). Protein abundance of both catalase and MnSOD were similar between all treatment groups (Figure 3B).

Enzymatic activities of catalase (Figure 4B) and SOD (Figure 4D) were similar between all treatment groups in STW. Transcript abundance of antioxidant enzymes in the STW (Figure 2B) supports the numerical increase observed in SOD activity in STW (Figure 4D), though it failed to reach statistical significance.

Inflammatory Response

We have previously shown that HS resulted in a systemic increase in LPS (43), which has the potential to trigger an inflammatory response in tissues with a TLR4 receptor, including skeletal muscle (24). Further, independent of systemic pro-inflammatory factors, oxidative stress can also initiate an inflammatory response (7, 25, 59). Hence, in heat stressed skeletal muscle two independent mechanisms may drive inflammatory signaling. To determine the extent to which skeletal muscle contributes to systemic inflammation we evaluated NF-κB pathway activation, a major pathway involved in inflammatory signaling. To assess inflammatory signaling we measured relative protein abundance of NF-κB (p65 subunit), activated phospho- NF-κB, the NF-κB inhibitor, IkB-α, and the pathway product IL-6 (11). We found that the relative abundance of NF-κB, phospho- NF-κB, IkB-α and IL-6 was similar between all treatment groups in STR (Figure 5A). Further supporting pathway quiescence, mRNA

transcript abundance of pathway products *TNF*, *IL15* and *IL1B* were similar between all groups in STR (Figure 6A). Of interest, in STR, relative protein abundance of TNF-α was increased 2.5 fold ($p<0.05$) after 1 and 3 days of HS compared to TN (Figure 5B).

In the STW, relative protein abundance of TNF-α, NF-κB and phospho- NF-κB were similar between all treatment groups (Figure 5B). IkB-α protein abundance was decreased by 40% ($p<0.05$) after 3 days of HS compared to TN (Figure 5B). Similarly, IL-6 protein abundance was decreased by approximately 60% after 1 and 3 days of HS ($p<0.05$) compared to TN (Figure 5B). *TNF* and *IL1B* transcript abundance were similar between all treatment groups, but *IL15* was significantly increased ($p<0.05$) after 3 days of HS (Figure 6B).

Discussion

Heat stress negatively impacts human health (10) and animal production (50), particularly in regard to muscle growth. Despite the broad, deleterious consequences of HS, little is known about how HS alters skeletal muscle physiology. It is necessary to improve this understanding so that mitigation strategies for human morbidity and mortality as well as compromised animal growth can be developed. In this investigation we tested the hypotheses that HS caused oxidative stress and increased inflammatory signaling in porcine skeletal muscle. Our data show that HS selectively caused free radical damage in oxidative muscle, but not glycolytic muscle. Counter to our hypothesis, we also found that HS did not initiate an inflammatory signaling response in porcine skeletal muscle in either muscle type.

Our finding of increased free radical injury is in good agreement with published reports in avian and amphibian skeletal muscle (2, 34, 35, 57). Previously, HS was found to increase the production of free radicals after 12, 16 and 18-hours (26, 35) of HS in chicken skeletal muscle. Further, the time of exposure resulting in that injury suggests a rapid onset of changes that led to a pro-oxidant intracellular environment, like in our investigation. We also found that oxidative injury was transient. The persistence of oxidative injury in the previous investigations, however, is unknown as only a single or early time points were included. Oxidative injury did persist following a 14-day HS protocol in chicken skeletal muscle (2). It is unclear though if this persistence of free radical injury is a result of the prolonged heating period, muscle type (avian compared to mammalian), or some other factor. In mammals, heat stressed dairy cows had increased oxidative stress levels in plasma (6), though the effect of on HS on skeletal muscle was not addressed. Clarity regarding the onset and persistence of oxidative damage is hindered by the surprising lack of similar studies in mammalian skeletal muscle.

The increase in free radical injury was well countered by corresponding changes in antioxidant enzyme expression and activity. Given the close timing of increased free radical damage following 1 day of HS and the corresponding increase in antioxidant enzymes, it raises the possibility of greater damage occurring at an earlier time point where injury and antioxidant enzyme expression may be uncoupled. Also, that we were unable to detect increased oxidant injury or increased antioxidant enzyme activity following 3 days of HS suggests that the rate of free radical production returned to TN conditions. Such a change is indicative of a significant shift in cellular physiology.

Of interest then is the source of free radical production. A common trigger of free radical production in skeletal muscle is a loss of Ca^{2+} homeostasis. Indeed, SERCA, a sarcoplasmic reticulum Ca^{2+} ATPase, function is decreased in heat stress conditions (12, 28, 45) potentially contributing to a loss of Ca^{2+} homeostasis. Increased free Ca^{2+} within a cell can decrease mitochondrial membrane potential (16), and lead to electron leakage, primarily in complexes I and III, ultimately culminating in increased ROS production (15, 20). Further, increased MnSOD expression is indicative of increased free radical production from the mitochondria (27). Also pointing to mitochondria as the source of free radicals is the observed metabolic shift toward increased reliance on glycolysis for ATP production (4, 55). This shift away from mitochondrial flux for ATP production may serve to mitigate production of these radicals. Finally, increased superoxide production (35-37) from heat stressed mitochondria isolated from avian skeletal muscle was previously reported along with associated changes in uncoupling protein expression (35) raising the possibility of a similar mechanism in mammalian skeletal muscle. Considering this evidence, we postulate that the mitochondria are the primary source of free radical production during HS in mammalian skeletal muscle, however, acknowledge that cytosolic sources of free radical production should also be considered.

Regardless of source, findings in this investigation and those cited above lead us to propose the following mechanism. We hypothesize that the pro-oxidant intracellular environment leads to oxidation of myoglobin-bound iron from Fe^{2+} (ferrous) to Fe^{3+} (ferric), which impairs its O_2 binding capacity. While sufficient 0_2 is likely delivered to

the muscle via circulating hemoglobin, 0_2 delivery to the mitochondria fails because of the oxidation state of myoglobin-bound iron. This, in turn, leads to cellular hypoxia, which leads to induction of HIF-1 and metabolic dysregulation. Indeed, increased abundance of HIF-1 and downstream activation has been previously reported in heat stressed C. elegans (51), mouse testes (42), and rat hearts (30). Evidence supporting a glycolytic shift has been previously discussed (4, 55). In future investigations we will measure the oxidation status of myoglobin in heat stressed skeletal muscle. In this proposed mechanism a transient increase in oxidative stress would be expected to have persistent effects via activation of HIF-1.

Though LPS, TNF-α and ROS were increased in these HS animals (43), these data indicate that HS did not lead to an increase in inflammatory signaling in porcine skeletal muscle. Given the lack of evidence that supports increased inflammatory signaling the noted increase in TNF-α protein abundance in STR (Figure 5) is puzzling. Speculatively, there are two likely sources for this additional TNF-α including delivery by the circulatory system or production by muscle cells. If indeed, TNF-α is entering skeletal muscle via the circulation, we would anticipate increased abundance in both STR and STW. This interpretation is confounded however, as oxidative muscle has a larger blood flow than glycolytic muscle (1, 19, 23) potentially allowing greater TNF-α accumulation in oxidative muscle. Alternatively, TNF-α may have been differentially produced by oxidative and glycolytic muscle. Given our data showing similar inflammatory signaling between HS and TN animals, this possibility appears less likely.

Nevertheless, increased TNF-α abundance may have resulted from early changes in inflammatory signaling that had abated by our first data point following 1 day of HS.

In summary, the first 24 hours of HS resulted in increased oxidative stress in the STR but not STW. This insult was quickly compensated by an antioxidant response, resulting in the resolution of oxidative damage by 72 hours. Further, selective increases in MnSOD and oxidative stress in the STR, but not the STW strongly suggest an involvement of the mitochondria in the HS response (27). However, contrary to our expectations, HS did not seem to play a role in the initiation of an inflammatory response in porcine skeletal muscle.

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Figure Legends

Figure 1. Heat stress increased oxidative injury in oxidative, but not glycolytic muscle. Oxidative stress was measured by quantifying the relative abundance of MDA modified proteins, a marker of lipid peroxidation. (A) MDA modified proteins increased 2.5 fold compared to TN after 1 day of HS in the STR (TN, n=6; 1 HS, n=5; 3 HS, n=5) (B) but remained unchanged in the STW (TN, n=6; 1 HS, n=5; 3 HS, n=4). * indicates significantly different from TN; $p<0.05$

Figure 2. Gene expression of antioxidant enzymes following heat stress. (A) HS increased gene expression of both *CAT* (TN, n=6; 1 HS, n=6; 3 HS, n=6) and *SOD2* (TN, n=6; 1 HS, n=5; 3 HS, n=6) following 1 day of HS, but not that of *SOD1* (TN, n=6; 1 HS, n=5; 3 HS, n=6) in the STR. Expression of all antioxidant enzymes was decreased after 3 days of HS compared to 1 day of HS in the STR. (B) HS increased *SOD2* gene expression after 1 day of HS compared to TN, though expression of all antioxidant enzymes was decreased by day 3 in the STW (TN, n=6; 1 HS, n=6; 3 HS, n=6). * indicates significantly different from TN; # indicates significantly different from 1-HS; $p<0.05$.

Figure 3. Protein expression of catalase and SOD following heat stress. (A) Catalase protein content was unchanged with HS in the STR. However, MnSOD protein content was increased after 1 and 3 days of HS in the STR. (B) Catalase and MnSOD protein content remain unchanged with HS in the STW. * indicates significantly different from TN; $p<0.05$.

Figure 4. Antioxidant enzyme activities following HS treatment in porcine skeletal muscle. (A) One day of HS increased catalase activity in STR (TN, n=6; 1 HS, n=5; 3 HS, n=6) (B) but not STW (TN, n=6; 1 HS, n=6; 3 HS, n=6) compared to TN. (C) Likewise, SOD activity was increased after 1 day of HS in the STR (TN, n=10; 1 HS, n=6; 3 HS, n=6) (D) but remained similar in STW (TN, n=10; 1 HS, n=6; 3 HS, n=6). * indicates significantly different from TN; # indicates significantly different from 1-HS; $p<0.05$.

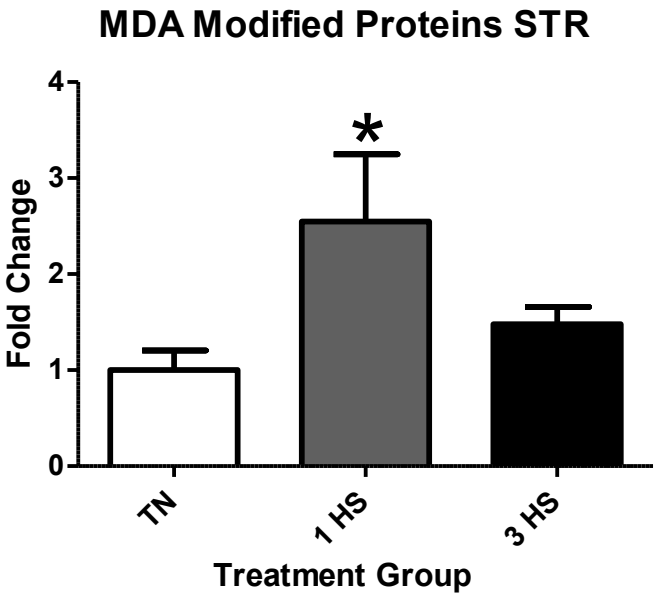
Figure 5. Protein expression of inflammatory signaling molecules in the NF-κB pathway. (A) In STR, IkB-α, NF-κB, phospho-NF-κB and IL-6 protein abundance were similar between all treatment groups in the STR, however TNF-α was increased 2.5 fold following one and three days of HS (n =5-6/group). (B) In the STW, TNF-α, NF-κB and phospho-NF-κB protein content remain unchanged, however, HS decreased IkB-

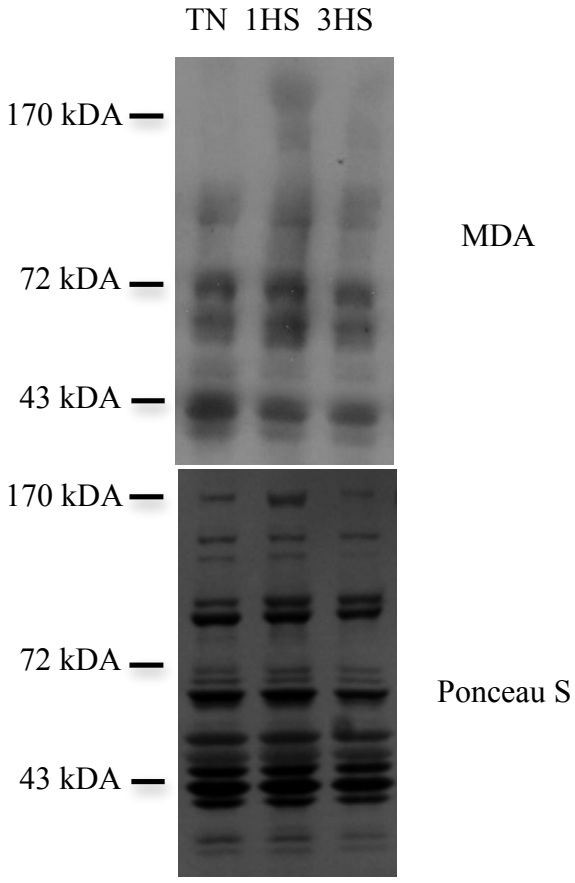
α and IL-6 protein content (n=5-6/group). * indicates significantly different from TN; $p<0.05$

Figure 6. Gene expression of inflammatory cytokines in skeletal muscle following HS. (A) Gene expression of *TNF*, *IL1B* and *IL15* was similar between all treatment groups in STR (n=4-5/group). (B) Gene expression of *TNF* and *IL1B*, was similar between all treatment groups, but *IL15* was increased after 3 days of HS in STW (n=4-5/group). * indicates significantly different from TN; $p<0.05$

Figure 1

A


MDA Modified Proteins STR
Fold Change
4
3
2
1
0
*
TN
1 HS
3 HS
Treatment Group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72 kDA
43 kDA
MDA
170 kDA
72 kDA
43 kDA
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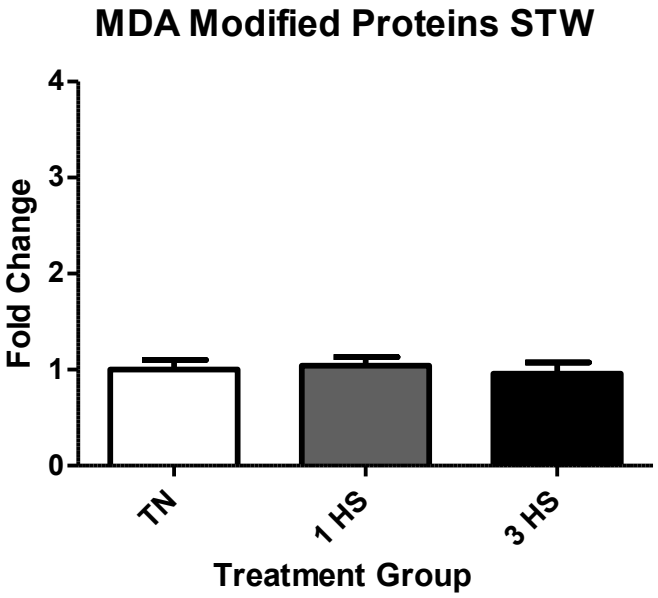
Figure 1 continued

B



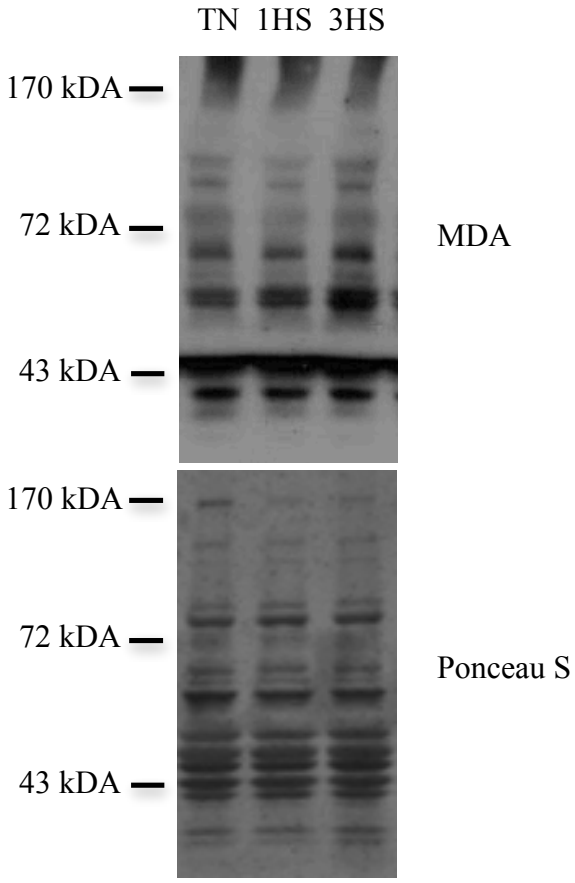


Figure 2

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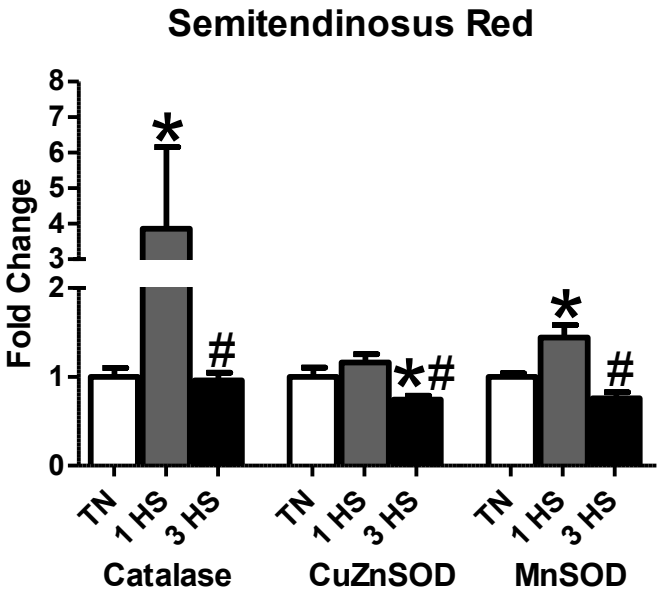
Semitendinosus Red
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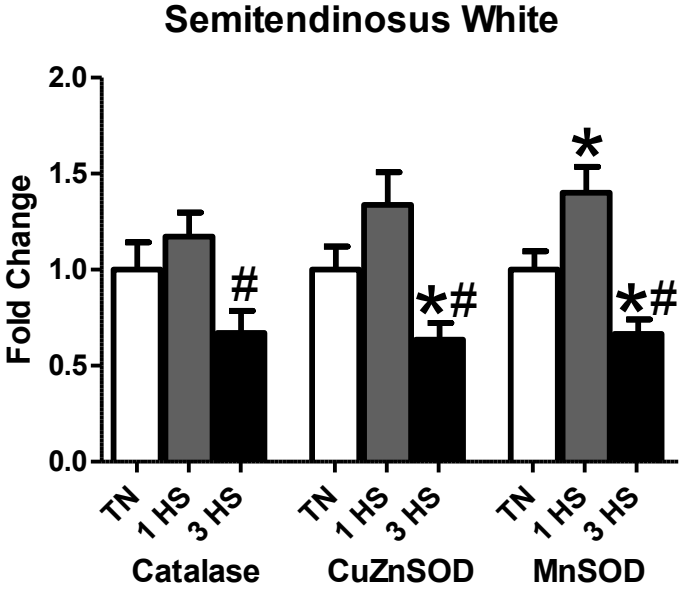
TN
1 HS
3 HS
Catalase
CuZnSOD
MnSOD

B


Semitendinosus White
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1.0
0.5
0.0
#
*#
*
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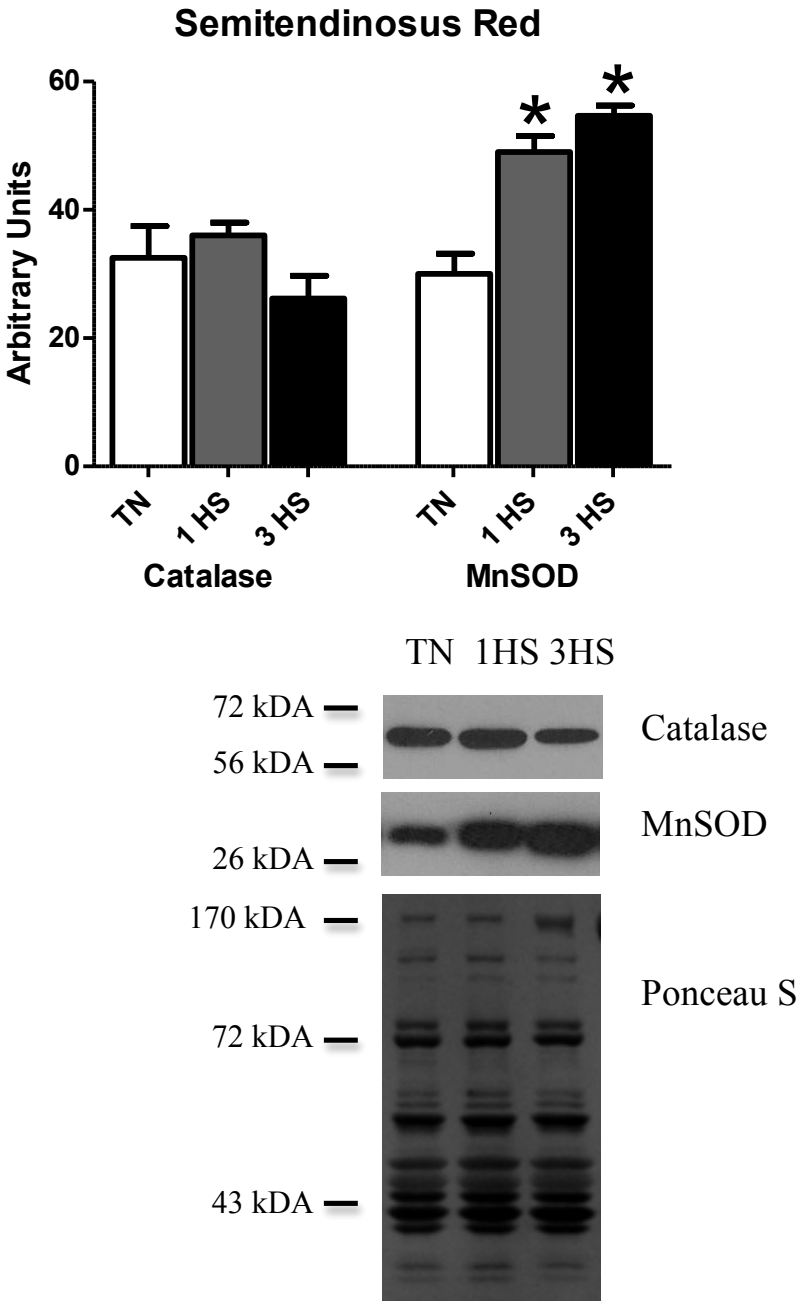
3 HS
TN
1 HS
3 HS
TN
1 HS
3 HS
Catalase
CuZnSOD
MnSOD

Figure 3

A


Semitendinosus Red
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*
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3 HS
TN
1 HS
3 HS
Catalase
MnSOD
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56 kDA
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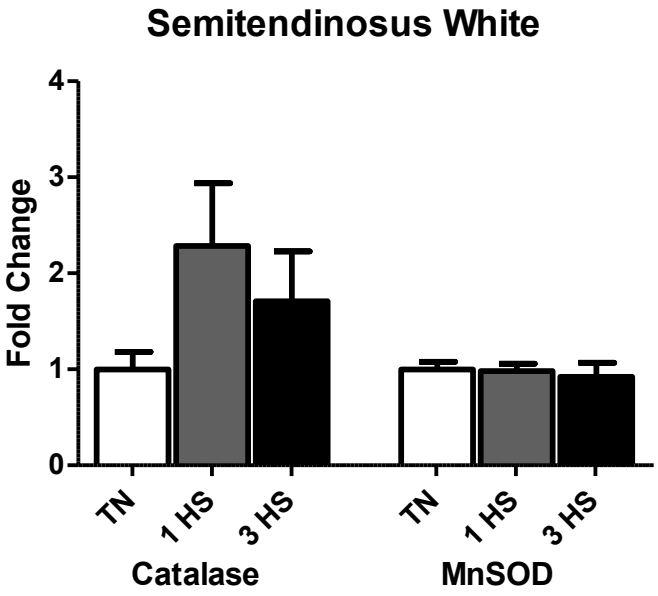
43 kDA
Catalase
MnSOD
Ponceau S

Figure 3 continued

B



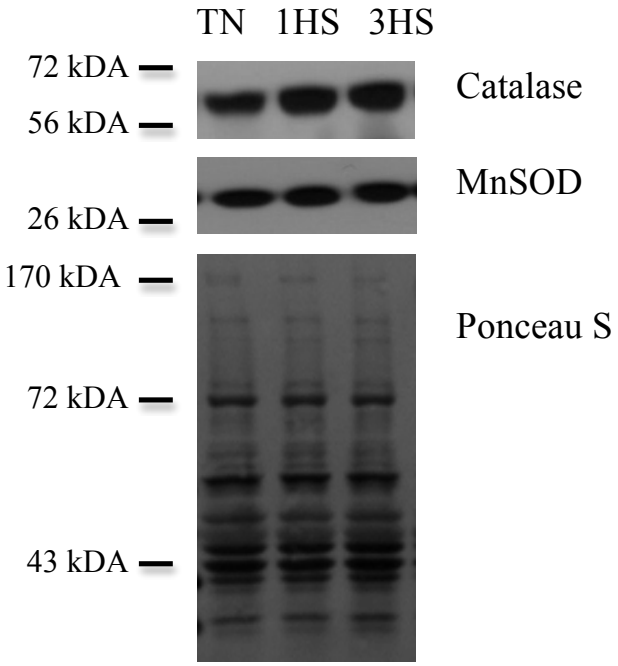


Figure 4

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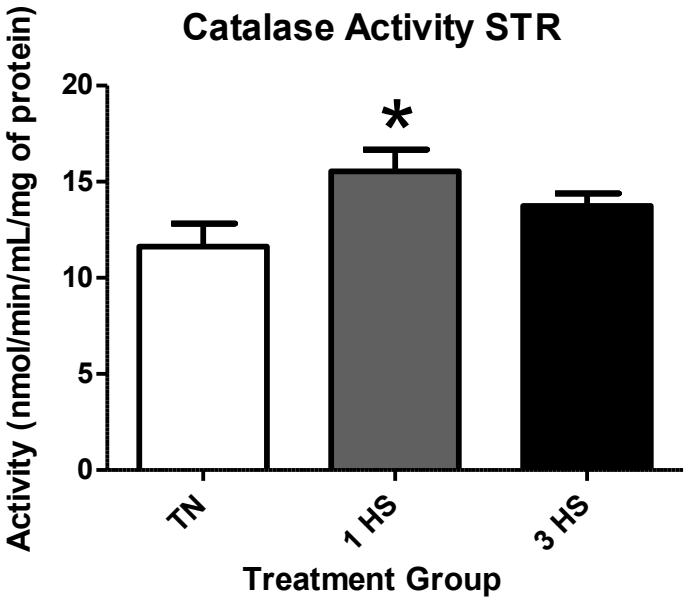

Catalase Activity STR
Activity (nmol/min/mL/mg of protein)
20
15
10
5
0
*
TN
1 HS
3 HS
Treatment Group

B

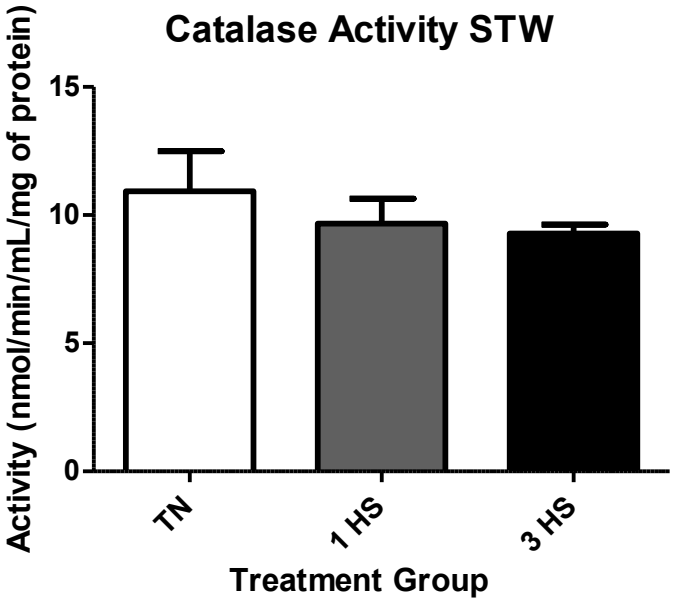
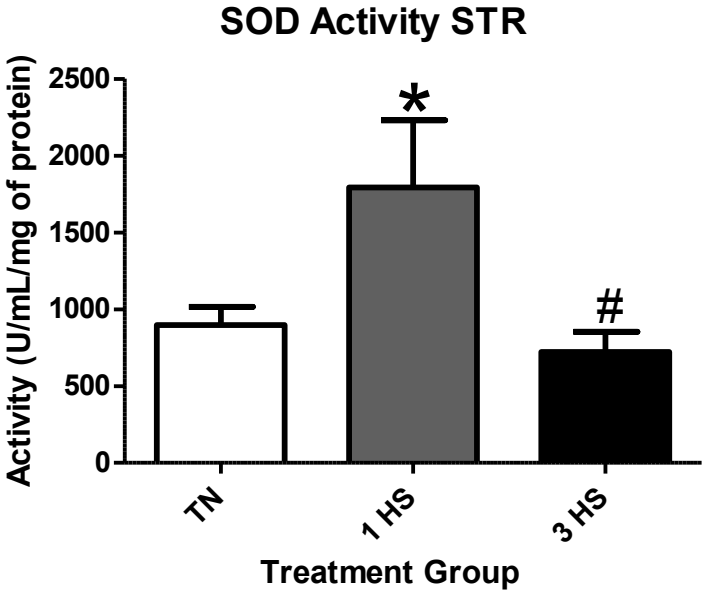

Catalase Activity STW
Activity (nmol/min/mL/mg of protein)
15
10
5
0
TN
1 HS
3 HS
Treatment Group

Figure 4 continued

C



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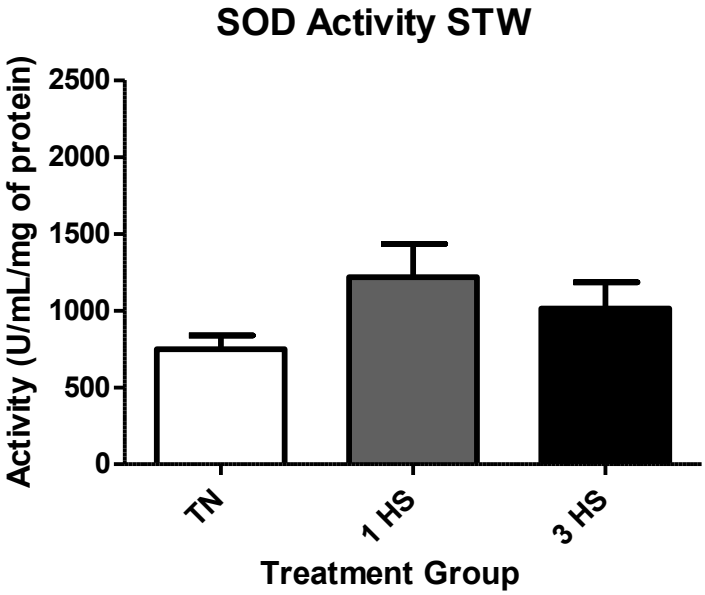
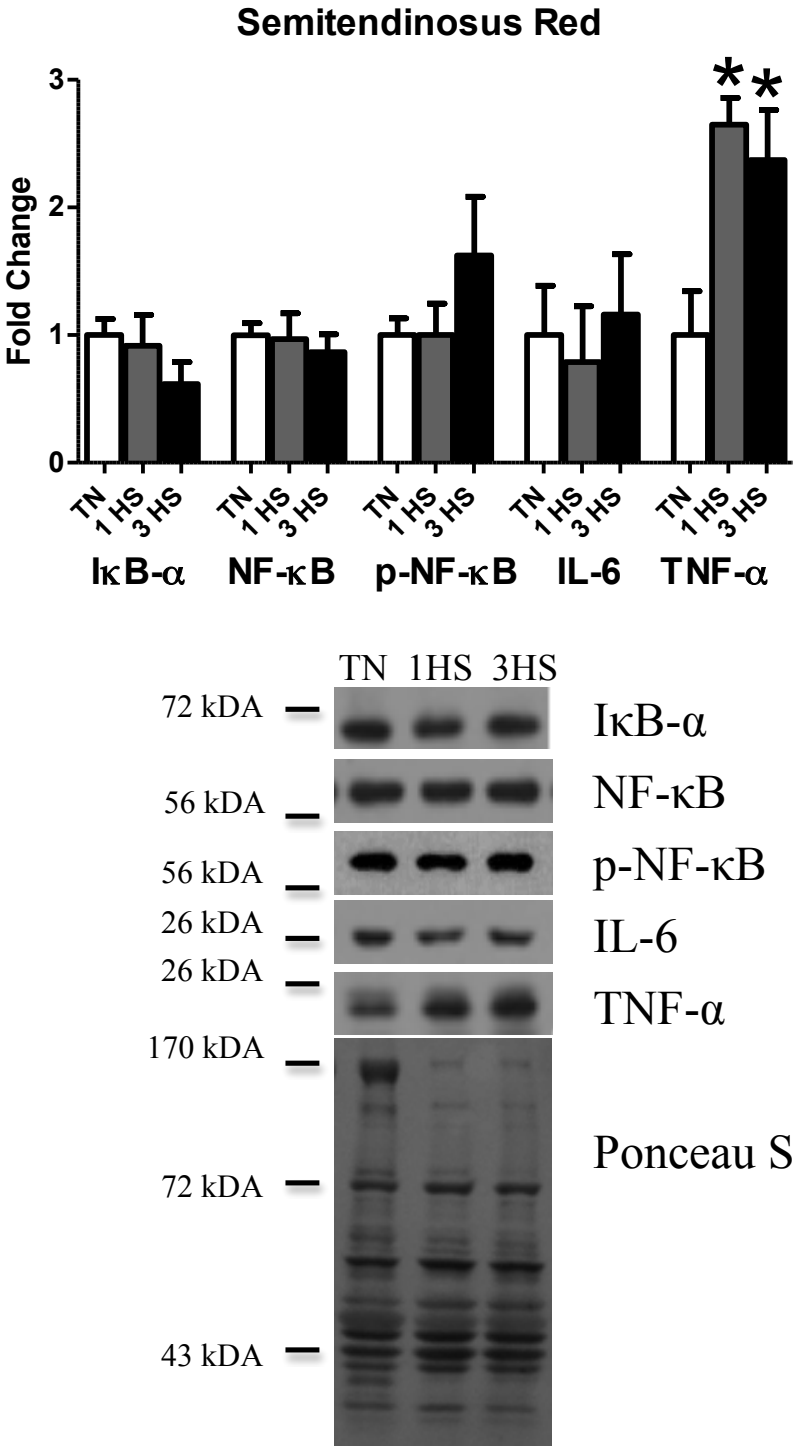


Figure 5

A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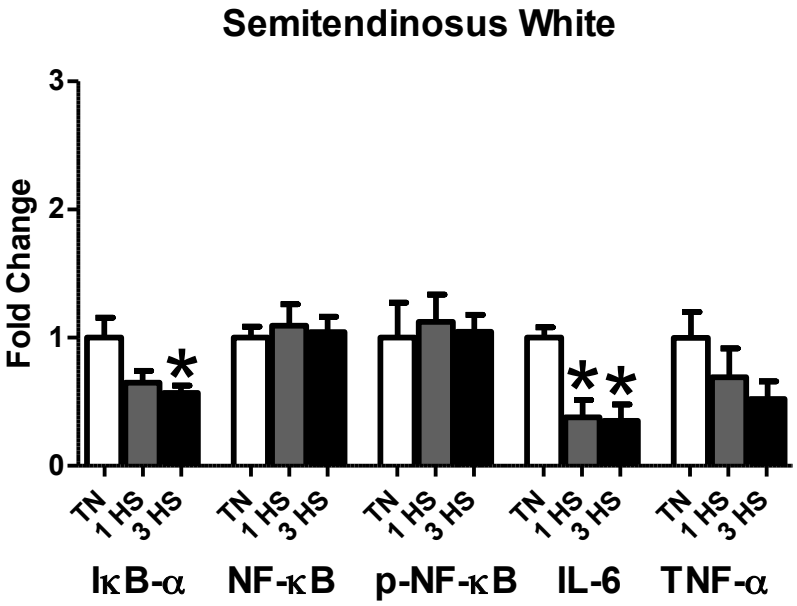
Fold Change
3
2
1
0
* *
TN
1 HS
3 HS
IκB-α
NF-κB
p-NF-κB
IL-6
TNF-α
TN 1HS 3HS
72 kDA
56 kDA
56 kDA
26 kDA
26 kDA
170 kDA
72 kDA
43 kDA
IκB-α
NF-κB
p-NF-κB
IL-6
TNF-α
Ponceau S

Figure 5 continued

B



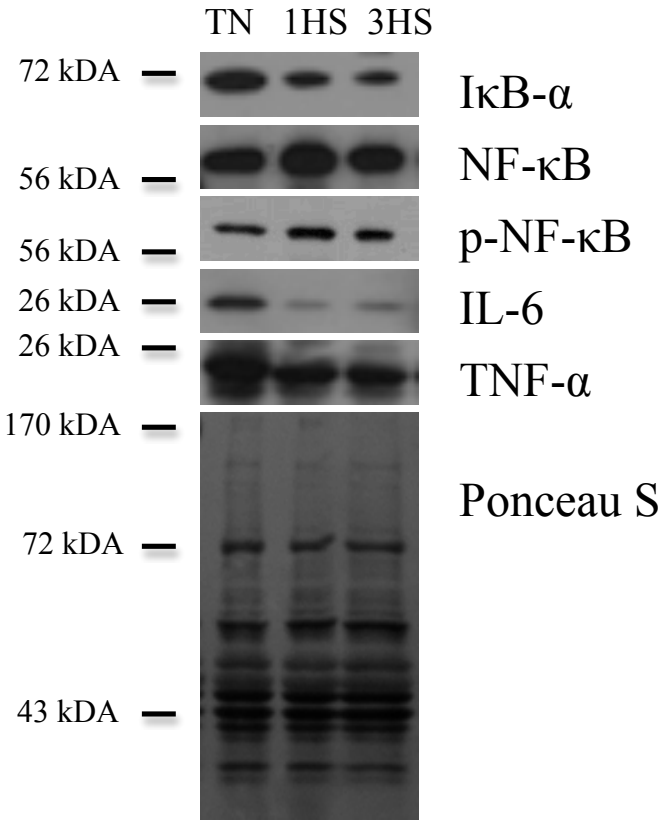
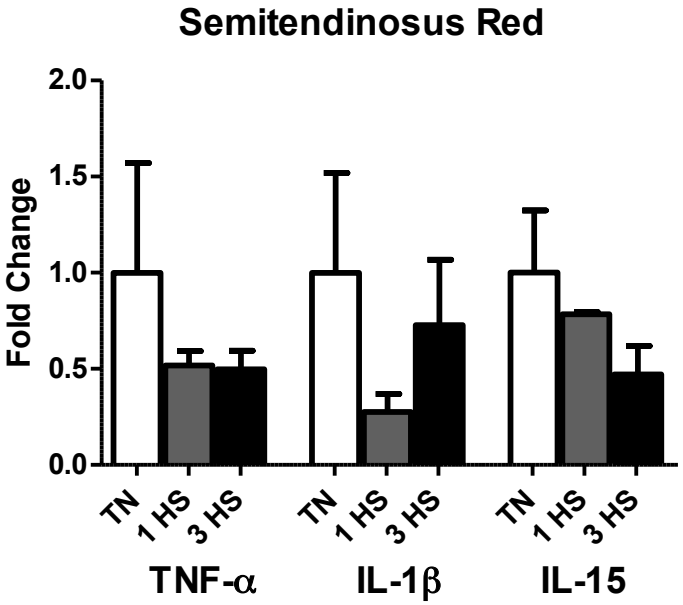


Figure 6

A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Fold Change
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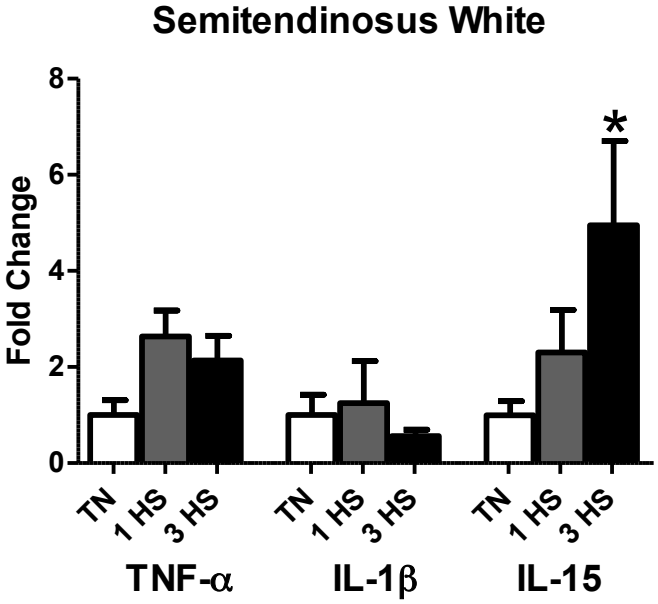
TNF-α
IL-1β
IL-15

B


Semitendinosus White
Fold Change
8
6
4
2
0
*
TN
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3 HS
TNF-α
IL-1β
IL-15

Table 1. Primer sequences used to measure relative mRNA abundance.

Gene of Interest	**Forward Primer**	**Reverse Primer**
TNF-α (*TNF*)	GCCCTTCCACCAACGTTTTC	TCCCAGGTAGATGGGTTCGT
IL-1β (*IL1B*)	AAGATAACACGCCCACCCTG	TGTCAGCTTCGGGGTTCTTC
IL-15 (*IL15*)	CAGAAGCAACCTGGCAGCACG	ACGCGTAACTCCAGGAGAAAGCA
Catalase (*CAT*)	CAGCTTTAGTGCTCCCGAAC	AGATGACCCGCAATGTTCTC
MnSOD (*SOD2*)	CGCTGAAAAAGGGTGATGTT	AGCGGTCAACTTCTCCTTGA
CuZnSOD (*SOD1*)	CGAGCTGAAGGGAGAGAAGA	AGTCACATTGCCCAGGTCTC

CHAPTER III

REDUCED FEED INTAKE DOES NOT CONTRIBUTE TO OXIDATIVE STRESS OR INFLAMMATORY SIGNALING IN PORCINE SKELETAL MUSCLE

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Sarah C. Pearce – Animal treatments, tissue collection, study design.
Delphine Gardan-Salmon – Data collection and analysis, tissue collection.
Nicholas K. Gabler – Study design, animal treatment, tissue collection, edited manuscript.
Jason W. Ross - Study design, animal treatment, tissue collection, edited manuscript.
Robert P. Rhoads - Study design, data collection, data analysis, edited manuscript.
Lance H. Baumgard - Study design, animal treatment, tissue collection, edited manuscript.
Steven M. Lonergan - Study design, edited manuscript.
Joshua T. Selsby - Study design, tissue collection, data analysis, data interpretation, edited manuscript.

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Abstract

Heat stress (HS) leads to multimillion-dollar losses annually in the swine industry. Greatly contributing to this cost is impaired muscle growth seen in HS animals. One of the mechanisms implemented by HS pigs in order to decrease their metabolic heat production is to reduce their feed intake. In many HS investigations distinguishing between the specific effects of HS and those caused or exacerbated by decreased feed intake confounds data interpretation. The purpose of this study was to determine the extent to which long term HS leads to oxidative stress and inflammatory signaling as well as quantify the contribution of decreased feed intake to these responses. To achieve this, crossbred gilts were subjected to thermoneutral (TN; 20°C) or HS (35°C) conditions for 7 days after which the semitendinosus was removed and divided into red (STR) and white (STW) portions. To account for reduced feed intake, another group of TN animals was pair-fed (PFTN) to the HS group. HS and PFTN animals had similar levels of MDA modified proteins when compared to the TN animals in the STR and STW. Transcript abundance of CuZnSOD was similarly decreased ($p<0.05$) in both the HS and PFTN group compared to TN in the STR and STW. Further, transcript abundance of MnSOD was decreased ($p<0.05$) in the HS and PFTN groups compared to TN in the STW. Enzymatic activity of catalase and SOD were similar between all treatment groups in both STR and STW. Neither HS nor pair feeding increased inflammatory signaling in the STR or STW. These results indicate that 7 days of HS and reduced feed intake down regulate antioxidant enzyme gene expression but do not lead

to oxidative stress or inflammatory signaling. Thus, other mechanisms must be contributing to the observed decrease in protein accretion.

Keywords: heat stress, oxidative stress, inflammation, reduced feed intake

Introduction

HS compromises animal agriculture production by negatively affecting growth, reproductive ability, and other important production traits, which costs the US livestock industry between $1.69 and $2.36 billion annually (22). In the swine industry specifically, HS results in $300 million losses annually due to heat related inefficiencies (22). Heat stress (HS) occurs when an animal is unable to regulate the ratio of heat being produced or absorbed to the amount of heat being dissipated (22). Heat stress occurrences are likely to increase in the next few years as as the Earth's average temperature has increased 0.7°C in the last decade and is predicted to continue increasing (14). Though reduced feed intake is partly responsible for the observed decreased in growth, losses are such that they are greater than expected by decreased feed intake alone (18, 24). Moreover, the mechanism by which HS leads to impaired muscle growth is largely unknown. Thus studying the effects of HS on pigs will greatly benefit livestock production.

ROS have been implicated with impaired protein synthesis (20, 27), and increased protein degradation (4, 10, 11, 21, 25). Indeed, previous studies have shown that between 12 hours (9, 13) and 9 days (1) of HS increases the production of reactive oxygen species (ROS) in avian skeletal muscle. Thus, increased ROS production and

oxidative stress may be one of the mechanisms by which HS results in impaired muscle growth and decreased protein accretion.

Another mechanism by which HS can result in decreased muscle growth is inflammation. It is well known that endotoxemia can activate inflammatory signaling. Given that HS pigs experience increased intestinal permeability (15, 16), it is likely that bacterial lipopolysaccharide (LPS) can enter the circulation and initiate an inflammatory response via NF-κB signaling (7). NF-κB signaling has been implicated with increased production of ubiquitin ligases, as well as muscle atrophy (3, 6). Importantly, skeletal muscle expresses receptors for LPS (toll-like receptors) (5) and can contribute to inflammatory signaling via NF-κB (23).

Therefore, the purpose of this study was to determine the extent to which long-term HS contributes to oxidative stress and inflammatory signaling in porcine skeletal muscle, and quantify how much of the response was due to reduced feed intake. We hypothesized that HS would result in oxidative stress and inflammatory signaling in porcine skeletal muscle, and that reduced feed intake would be partly, but not solely, responsible for elevations in these pathways.

Materials and Methods

Study Design and Animal Treatments

All animal experiments were approved by the Iowa State University Institutional Animal Care and Use Committee. Previous data from these animals and a detailed study design have been previously reported (15). Briefly, crossbred gilts (35±4 kg BW; 5-6/group) were held at thermoneutral (TN) conditions (20 ± 1°C; 35-50% relative humidity) or exposed to constant heat stress (HS) (35 ± 1°C; 20-35% relative humidity) for a period of 7 days. A group of TN animals was pair-fed to match daily intake of the HS group. Animals were sacrificed by the captive bolt technique followed by exsanguination at the end of treatment. At this time, semitendinosus (ST) muscle was removed and carefully divided into red (STR) and white (STW) muscle, and then frozen in liquid nitrogen for further analyses.

Protein Abundance

To assess protein abundance, muscle samples were homogenized and prepared as previously described (19). Briefly, whole muscle homogenates were diluted in loading buffer to 4mg/mL, and 40 μg of protein were separated and transferred into a nitrocellulose membrane. Membranes were blocked for an hour in 5% dehydrated milk TTBS and exposed to primary antibody overnight at 4°C in 1% dehydrated milk TTBS solution as follows: Malondialdehyde (Abcam; primary 1:5000, cat. no ab27642, secondary 1:2000), NF-κB p-65 (Abcam; primary 1:1000, cat. no ab7970, secondary 1:2000), IL-6 (Abcam; primary 1:1000, cat. no ab6672, secondary 1:2000), phospho-NF-κB p65 (Thermo Scientific; primary 1:1000, cat. no MA5-15160, secondary 1:2000),

TNF-α (Abcam; primary 1:1000, cat. no ab6671, secondary 1:2000), IκB-α (Santa Cruz Biotechnology; primary 1:1000, cat. no SC-371, secondary 1:2000). Membranes were washed 3 times for 10 min with TTBS and exposed to secondary antibody for an hour at room temperature in 1% dehydrated milk TTBS solution. After 3 more 10 min washes in TTBS, detection was performed by enhanced chemiluminescence and X-ray film. X-ray film was then scanned and blot signal was quantified through the use of Kodak software (Rochester, NY). All membranes were stained with Ponceau S to assure equal loading.

mRNA Transcript Abundance

mRNA transcript abundance was measured as previously described (19). Briefly, muscle was homogenized with TRIzol reagent following manufacturers' instructions. RNA was DNAse treated to remove DNA contamination. Equal amounts of RNA were then reverse transcribed and gene expression was measured using SYBR green through qRT-PCR. Transcript abundance was analyzed by the delta CT method, and fold change calculated from the delta delta CTs with the use of 18s a s the control gene. mRNA transcript abundance data are presented as fold changes relative to TN. Sequences of primer pairs have been previously reported (19).

Enzymatic Activities

Catalase and total superoxide dismutase (SOD) activity were measured according to manufacturer instructions (Catalase Assay Kit, Cayman Chemical Company, Item No. 707002; Super Oxide Dismutase Assay Kit, Cayman Chemical Company, Item No. 706002).

Statistics

To determine the extent to which heat stress and reduced feed intake altered variables over time data from TN, HS, and PFTN animals were compared using an ANOVA followed by a Newman-Keuls post hoc test. To determine statistical significance, α level was set at $p < 0.05$. Values are reported as means ± SE unless otherwise noted.

Results

Oxidative Stress

To determine the extent to which heat stress caused free radical damage in skeletal muscle, abundance of proteins modified by malondialdehyde (MDA), which is a marker of lipid peroxidation, was measured in STR and STW. In both STR and STW, relative content of proteins that contained MDA adducts was similar between all treatment groups (Figure 1A and 1B).

In order to assess if the absence of oxidative injury was due to an effective antioxidant response, we measured mRNA transcript abundance of select antioxidant enzymes. In STR, transcript abundance of catalase and MnSOD was similar between all treatment groups (Figure 2A). However, both HS and reduced feed intake significantly decreased ($p<0.05$) transcript abundance of CuZnSOD compared to TN. Moreover, the PFTN group was significantly decreased ($p<0.05$) compared to the HS group. In the STW, transcript abundance of catalase was similar between all treatment groups, though that of CuZnSOD and MnSOD was significantly decreased in the HS and PFTN groups compared to TN (Figure 2B). Enzymatic activities of catalase and total SOD were also similar between all treatment groups in both the STR and the STW (Figure 3).

Inflammatory Response

We have previously shown that HS resulted in a systemic increase in LPS in HS pigs (15), which has the capability of initiating an inflammatory response in skeletal muscle (7). To examine the extent to which skeletal muscle contributes to systemic inflammation we assessed NF-κB pathway activation. We found that the relative protein abundance of NF-κB, phospho- NF-κB, IkB-α, IL-6 and TNF-α was similar between all treatment groups in STR (Figure 4A). In the STW, relative protein abundance of TNF-α, NF-κB, phospho- NF-κB and IkB-α were similar between all treatment groups (Figure 4B). IL-6 protein abundanec was decreased by 60% ($p<0.05$) after HS compared to TN and 30% as a result of reduced feed intake compared to TN (Figure 4B).

Discussion

HS leads to billion dollar losses in animal production (22), particularly associated with impaired muscle growth. However, much is still unknown about how HS alters skeletal muscle physiology. In order to devise new ways to mitigate these deleterious effects of HS, it is necessary to increase our understanding of the effects of HS on skeletal muscle. In this investigation we tested the hypothesis that long term HS, with a partial contribution from reduced feed intake, would cause oxidative stress and increase inflammatory signaling in porcine skeletal muscle. Contrary to our expectations, these data show that neither HS nor reduced feed intake increased oxidative stress or inflammatory signaling in porcine skeletal muscle. Further, we also found that reduced feed intake was responsible for the majority of the responses associated with HS in the measured variables.

Despite our previous finding of a transient increase in oxidative stress (19) our expectation in this investigation was that chronic HS would lead to free radical injury. This seemed particularly likely given such findings in chronically heated avian muscle (1, 12, 13) as well as increased oxidative stress in plasma measured in chronically HS cattle (2). The persistence of an apparent lack of free radical injury without an increase in antioxidant enzyme activities further substantiates the notion of a fundamental change in muscle cell physiology such that processes leading to increased oxidant production are down-regulated. This hypothesis is also supported by the down regulation of SOD transcript abundance in STR and STW, which are generally induced in response to superoxide production (2, 8). Further, this observation also represents a likely

distinction between chronic HS in avian and mammalian skeletal muscle. From a whole animal perspective it is interesting to note increased oxidative stress in plasma following chronic HS indicating an active source of radical production, however, given these data skeletal muscle does not appear to be that source.

Though LPS was increased in these animals (15), these data suggest that neither HS nor reduced feed intake led to an increase in inflammatory signaling in porcine skeletal muscle. This finding is consistent with our previous report following a shorter-term HS. Given the entirety of the data set, the reduction in IL-6 protein abundance in STW likely is spurious rather than indicative of decreased inflammatory signaling. Alternatively, the selective reduction in IL-6 protein abundance in STW but not STR could point toward fiber type-specific responses to chronic HS, as previously reported in amphibian muscle (26).

In summary, 7 days of HS did not affect redox balance or inflammatory signaling in porcine skeletal muscle. Further, reduced feed intake does not contribute to oxidative injury or initiation of inflammatory signaling in porcine skeletal muscle. Thus, other possible pathways leading to decreased protein synthesis and/or increased degradation should be considered in order to explain the reductions in muscle mass that are not explicable by reduced feed intake alone.

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Figure Legends

Figure 1. Oxidative stress remains unchanged with heat stress and reduced feed intake. Neither heat stress nor reduced feed intake alters oxidative injury in porcine skeletal muscle. Oxidative stress was measured by quantifying the relative abundance of MDA modified proteins. (A) MDA modified proteins are similar between all treatment groups in the STR (TN, n=6; 7 HS, n=5; PFTN, n=5) (B) as well as in the STW (TN, n=6; 7 HS, n=5; PFTN, n=5).

Figure 2. mRNA transcript abundance of antioxidant enzymes following heat stress and reduced feed intake. (A) Catalase and MnSOD transcript abundance remained

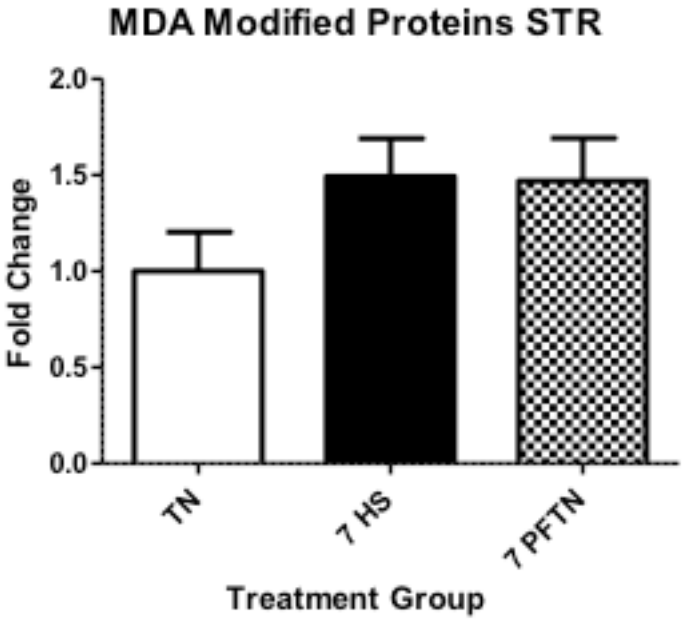
unchanged as a result of HS and reduced feed intake (TN, n=6; 7 HS, n=6; PFTN, n=6), though CuZnSOD (TN, n=6; 7 HS, n=6; PFTN, n=6) was suppressed following HS and reduced feed intake in the STR. The PFTN group was further suppressed compared to the HS group. (B) HS and reduced feed intake suppressed transcript abundance of both MnSOD and CuZnSOD compared to TN, though expression catalase remain unchanged in the STW (TN, n=6; 7 HS, n=6; PFTN, n=6). * indicates significantly different from TN; † indicates significantly different from 7 HS; $p<0.05$.

Figure 3. Antioxidant enzyme activities following HS treatment in porcine skeletal muscle. (A) HS and reduced feed intake had no effect in antioxidant enzyme activity of catalase (TN, n=6; 7 HS, n=6; PFTN, n=6) in STR (B) or STW (TN, n=6; 7 HS, n=6; PFTN, n=6) compared to TN. (C) Likewise, SOD activity was similar between all treatment groups in the STR (TN, n=6; 7 HS, n=6; PFTN, n=6) (D) and the STW (TN, n=6; 7 HS, n=6; PFTN, n=6).

Figure 4. Protein abundance of inflammatory signaling molecules in the NF-κB pathway. (A) In STR, IkB-α, NF-κB, phospho-NF-κB, IL-6 and TNF-α protein abundance were similar between all treatment groups in the STR (n =5-6/group). (B) In the STW, IkB-α, NF-κB, phospho-NF-κB and TNF-α protein content remain unchanged, however, HS and reduced feed intake decreased IL-6 protein content (n=5-6/group). * indicates significantly different from TN; $p<0.05$

Figure 1

A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TN
7 HS
7 PFTN
Treatment Group

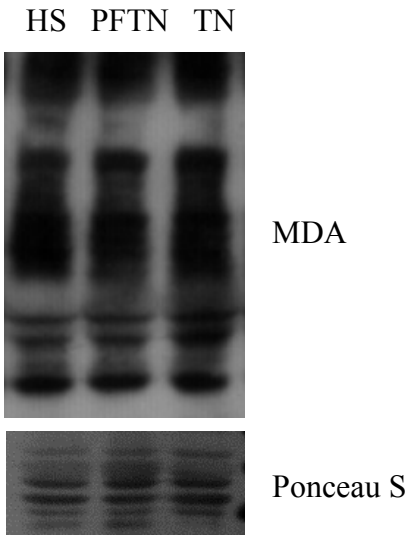
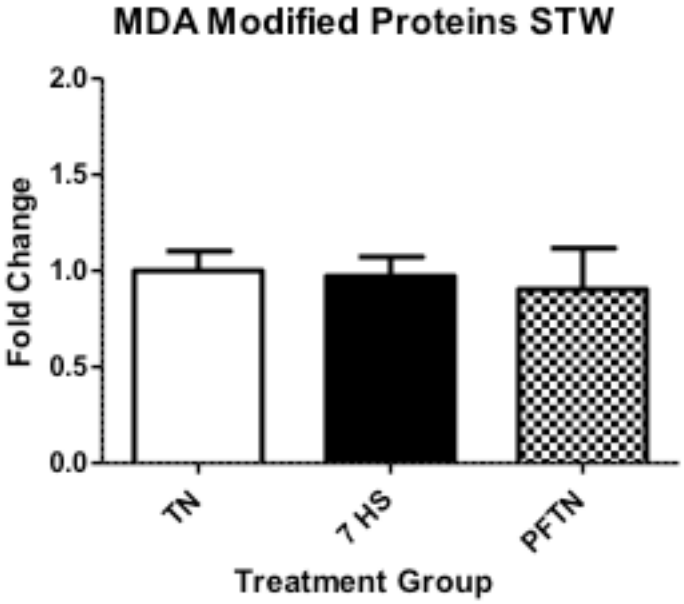

HS PFTN TN
MDA
Ponceau S

Figure 1 continued

B



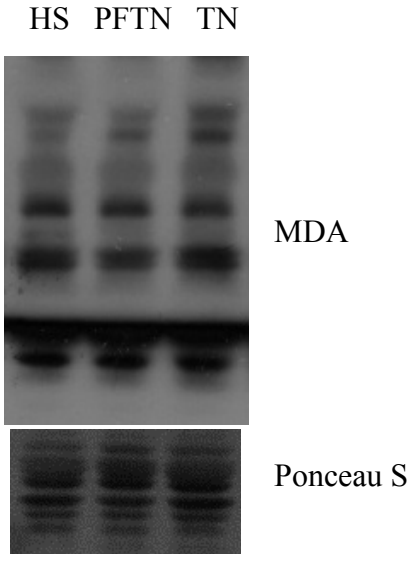
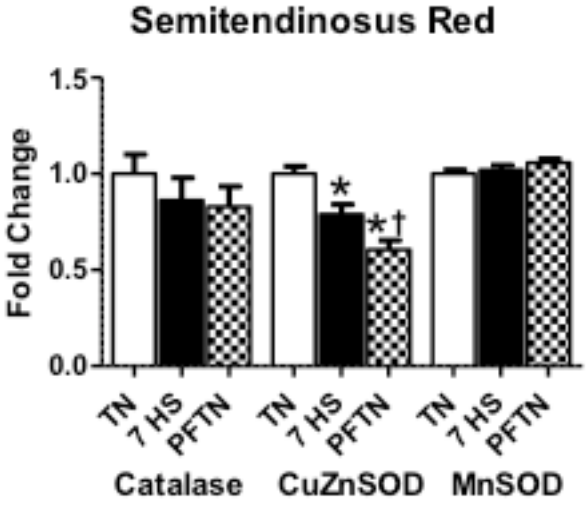


Figure 2

A


Semitendinosus Red
Fold Change
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*†
TN
7 HS
PFTN
Catalase
CuZnSOD
MnSOD

B

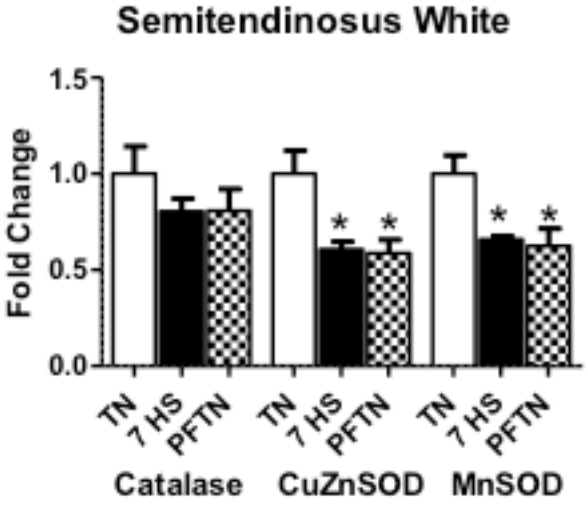
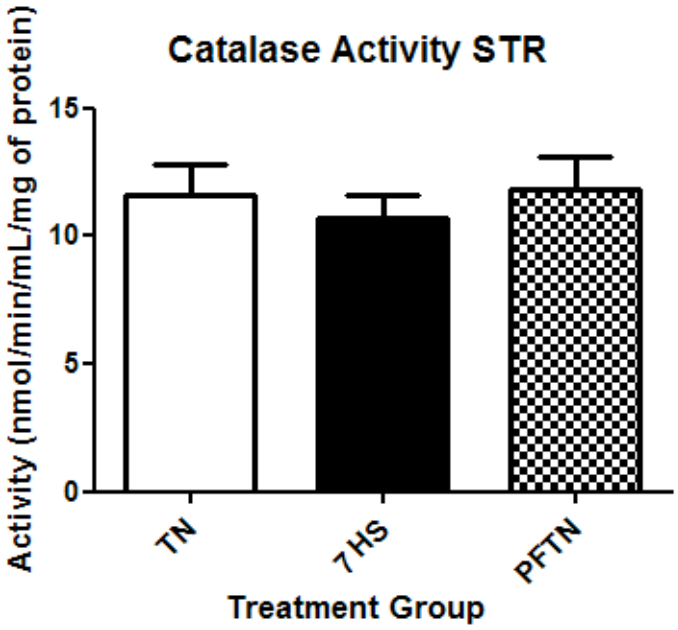

Semitendinosus White
Fold Change
1.5
1.0
0.5
0.0
*
*
*
*
TN
7 HS
PFTN
Catalase
CuZnSOD
MnSOD

Figure 3

A


Catalase Activity STR
Activity (nmol/min/mL/mg of protein)
15
10
5
0
TN
7HS
PFTN
Treatment Group

B

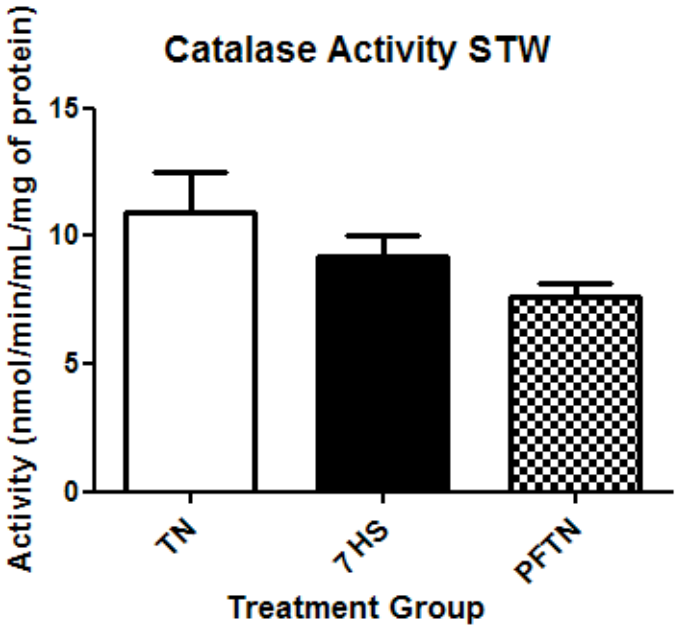
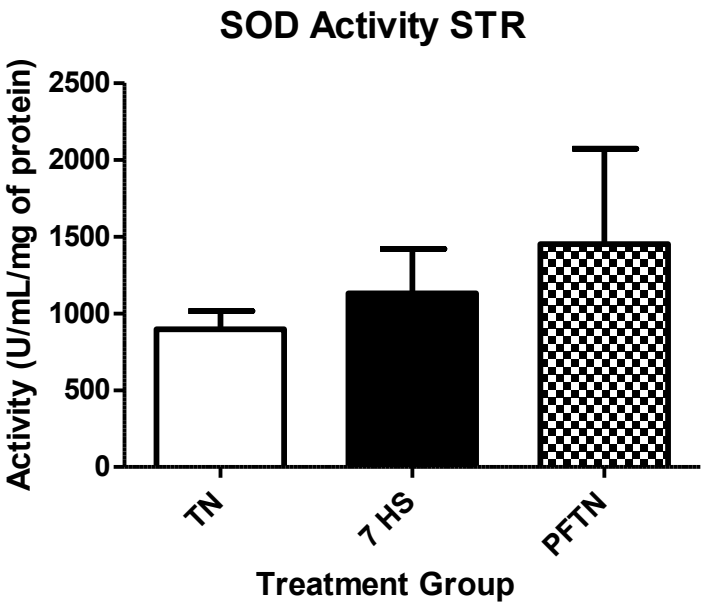

Catalase Activity STW
Activity (nmol/min/mL/mg of protein)
15
10
5
0
TN
7HS
PFTN
Treatment Group

Figure 3 continued

C



D

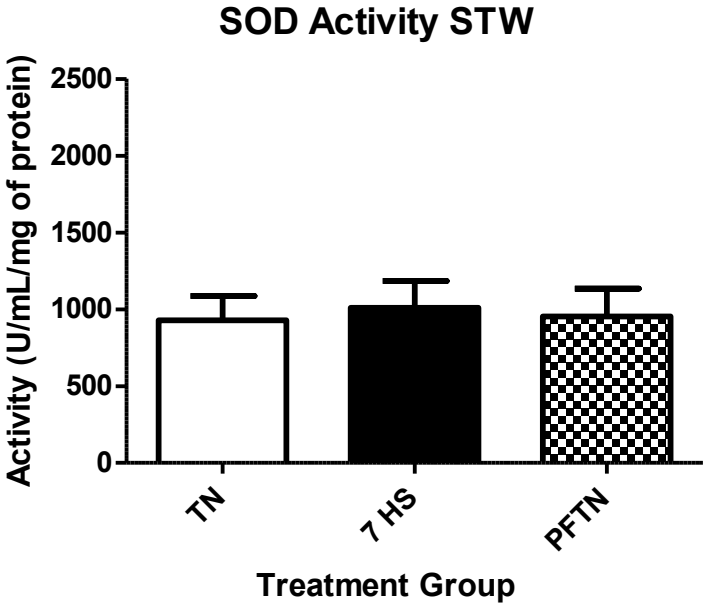
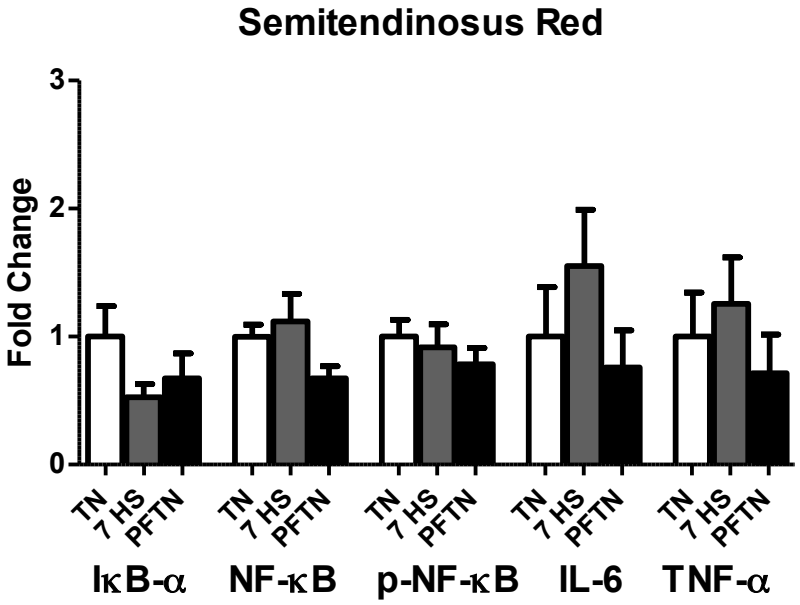


Figure 4

A


Semitendinosus Red
Fold Change
3
2
1
0
TN
7 HS
PFTN
IκB-α
NF-κB
p-NF-κB
IL-6
TNF-α

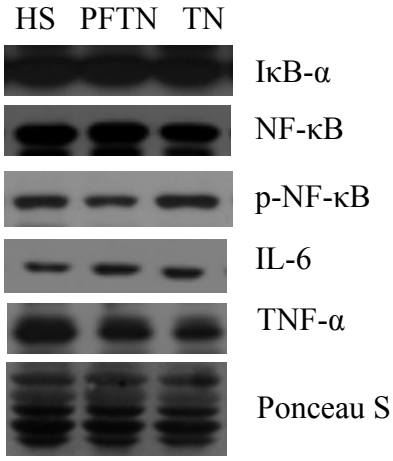
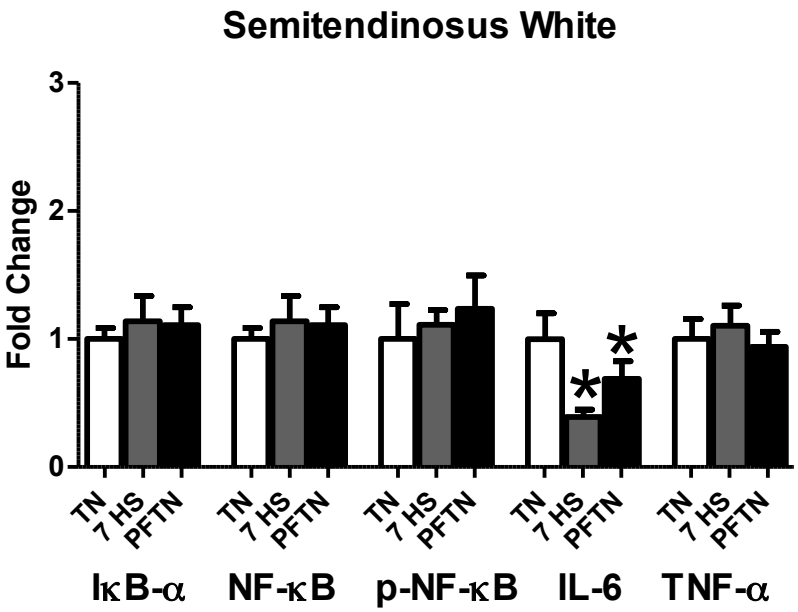
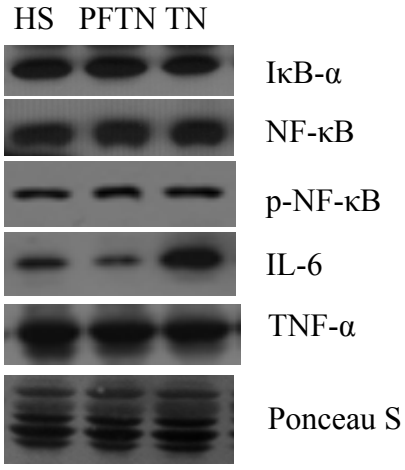

HS
PFTN
TN
IκB-α
NF-κB
p-NF-κB
IL-6
TNF-α
Ponceau S

Figure 4 continued

B





CHAPTER IV

INTEGRATIVE SUMMARY

Heat-related illnesses claim more lives than any other weather related illness in the US annually (60). In addition to human mortalities, agricultural animals experience impaired growth rate, decreased reproductive ability and undesirable production traits as a result of HS. Hence, HS is a burden, both for human health (16) and animal production (78), particularly in regard to muscle growth. Still, there is much to learn about how HS alters skeletal muscle physiology in order to develop mitigation strategies for human morbidity and mortality as well as compromised animal growth.

In our investigation we measured a transient increase in free radical damage in oxidative muscle, but not glycolytic muscle. The timeline of the injury suggests a rapid onset of changes that led to a pro-oxidant intracellular environment. The increase in oxidative stress was rapidly neutralized by a subsequent increase in antioxidant enzyme expression and activity. Because most of the observed changes in free radical damage and antioxidant enzymes are observed in the 24 hour time point, it is possible that greater damage occurred at an earlier time point where injury and antioxidant enzyme expression may be uncoupled. Of interest, following three days of HS and persisting to seven days of HS oxidant damage and antioxidant enzyme expression and activity were similar to TN animals. These observations are suggestive of a fundamental shift in cellular metabolism as there does not appear to be increased oxidant production that must be met with an antioxidant response. That this continued to seven days indicates a long-term adaptation rather than an acute change.

It seems likely that this change may be caused by decreased mitochondrial flux. MnSOD, a mitochondrial antioxidant enzyme, was particularly affected by HS, leading us to believe that mitochondria are the primary source of free radicals during HS. Further contributing to this idea, oxidative injury was only seen in oxidative muscle, which contains more mitochondria than glycolytic muscle, as mitochondria is their primary source of energy. Additionally, other researchers have reported a metabolic shift toward increased reliance on glycolysis for ATP production (5, 89), possibly in an attempt to reduce metabolic heat production.

We also showed that HS does not initiate an inflammatory signaling response in porcine skeletal muscle. Further, we demonstrated that reduced feed intake by itself does not contribute to oxidative stress or inflammatory signaling in porcine skeletal muscle. Thus, other protein synthesis and protein degradation mechanisms need to be considered to determine their contribution to the observed decrease in protein accretion.

In summary, the first 24 hours of HS resulted in increased oxidative stress in oxidative muscle. This insult was quickly compensated by an antioxidant response, resulting in the resolution of the stress by 72 hours. Further, selective increases in MnSOD and oxidative stress in the STR, but not the STW strongly suggest an involvement of the mitochondria in the HS response (46). However, contrary to what we expected, HS did not seem to play a role in the initiation of an inflammatory response in porcine skeletal muscle. Additionally, reduced feed intake did not contribute to oxidative stress or an inflammatory response in porcine skeletal muscle.

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